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Peptidyl prolyl cis-trans isomerase activity of cyclophilin A in functional homo-oligomeric receptor expression

(cyclosporin A/molecular chaperone/α7 nicotinic receptor/5HT$_3$ receptor/protein folding)

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ABSTRACT **The functional expression of homo-oligomeric α7 neuronal nicotinic and type 3 serotonin receptors is dependent on the activity of a cyclophilin. In this paper we demonstrate that the mechanism of cyclophilin action during functional homo-oligomeric receptor expression in *Xenopus* oocytes is distinct from the calcineurin-dependent immunosuppressive mechanism by showing that a nonimmunosuppressive analog of cyclosporin A (CsA), SDZ 211–811, reduces functional receptor expression to the same extent as CsA. The cytoplasmic subtype of cyclophilin, cyclophilin A (CyPA), appears to be required for functional receptor expression. This is because overexpression of CyPA and a CyPA mutant that is deficient in CsA binding activity reverses CsA-induced reduction in functional receptor expression. The mechanism of action of CyPA is likely to involve its prolyl isomerase activity because a mutant CyPA with a single amino acid substitution (arginine 55 to alanine) that is predicted to produce a 1000-fold attenuation in isomerase activity fails to reverse the cyclosporin A effect. Our data also suggest that CyPA does not form a stable complex with receptor subunits.**

The oligomeric assembly of neurotransmitter-gated ion channels is likely to be a complex multi-step process involving the participation of foldases and molecular chaperones (for review, see ref. 1). Functional homo-oligomeric α7 neuronal nicotinic (nAChR) and type 3 serotonin (5HT$_3$R) receptor expression is dependent on a cyclosporin A (CsA)-binding protein (2). CsA-binding proteins or cyclophilins (CyPs) are a family of immunophilins that are present in multiple cellular compartments such as the cytoplasm, the endoplasmic reticulum (ER), the mitochondria, and the extracellular space (for review, see ref. 3). They catalyze the cis-trans isomerization of peptide bonds between the carboxyl end of an amino acid and the amino end of a proline within a polypeptide chain (4, 5). Several proteins, including collagen (6), transferrin (7), and *Drosophila* rhodopsin (8, 9), have been shown to be the substrates of CyPs *in vivo*. It is thought that CyPs act either as peptidyl prolyl isomerases (PPIases) or as molecular chaperones to assist in the folding of these molecules (10). In addition to these functions, they also serve as cellular receptors that mediate the immunosuppressive actions of CsA by combining with it to inhibit calcineurin whose activity is required for T cell activation (11).

The recent discovery in our laboratory that a CyP is required for the functional expression of homo-oligomeric but not the hetero-oligomeric muscle-type nicotinic acetylcholine receptors lends support to the view that these molecules may play a wider but a more selective role in the folding and assembly of oligomeric cell surface molecules (2). This finding also adds to the growing evidence that the functional stability of other oligomeric ion channels such as the ryanodine calcium release channel (12, 13) and the inositol trisphosphate receptor (14) might be dependent on the activity of another immunophilin, the 12-kDa FK506-binding protein (FKBP-12). FKBP-12 is also a PPIase (15), and is also thought to function as a chaperone (16).

We have shown previously that CsA causes a significant reduction in the surface expression of the α7 neuronal nicotinic receptor in *Xenopus* oocytes. This reduction is not due to blockade of transcription or translation of this protein but appears to be due to some interference with maturational mechanisms such as folding and assembly. In this paper we attempt to understand the mechanism underlying this effect by establishing the molecular identity of the CyP mediating the CsA action and by determining the nature of the CyP activity that is involved in this process. Specifically, we address the following four questions related to actions of CsA on the functional expression of homo-oligomeric receptors in *Xenopus* oocytes: (*i*) Is the action of CsA mediated through the calcineurin-dependent mechanism that is involved in immunosuppression? (*ii*) Which form of CyP is responsible for the CsA effect? (*iii*) Does the action of CsA on functional receptor expression result from the selective blockade of the PPIase activity of this CyP? (*iv*) Does this CyP form a stable complex with the α7 receptor?

MATERIALS AND METHODS

Expression in *Xenopus* Oocytes. We introduced the α7 nicotinic and 5HT$_3$ receptors into *Xenopus* oocytes by injecting the eukaryotic expression vectors pcDNAI/AMP (Invitrogen) or pCDM6XL (a gift from A. Mariq and D. Julius, University of California, San Francisco) containing the respective subunit cDNAs using standard techniques. Rat brain CyPA, PC12 cyclophilin B (CyPB) and CyPA mutant cDNAs, CyPA(W121F), CyPA(R55A), and CyPAhis were coexpressed at 1:1 ratio with the receptor subunit cDNAs. The latter cDNAs were also contained in the pcDNAI/AMP vector. In CyP coexpression experiments, luciferase cDNA in pcDNAI/AMP vector was coexpressed with subunit cDNA (1:1 ratio) as control. All cDNA constructs were made in our laboratory. Functional expression was assayed electrophysiologically by performing two-electrode voltage-clamp recordings of agonist-induced currents. Near maximal currents were induced by ≈3- to 5-sec application of 200–500 μM of nicotine and 10 μM of 5HT to α7 and 5HT$_3$ receptors, respectively. CsA or CsA analog treatment involved incubating oocytes in 2–30

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Abbreviations: CsA, cyclosporin A; CyPA, cyclophilin A; CyPB, cyclophilin B; 5HT$_3$R, type 3 serotonin receptor; nAChR, nicotinic acetylcholine receptor; FKBP-12, 12-kDa FK506-binding protein; PPIase, peptidyl prolyl isomerase; ER, endoplasmic recticulum.

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μM concentrations (or vehicle control, 0.1% ethanol) in Barth's saline for 4–7 days. The CsA analog SDZ 211–811 was obtained as a gift from R. Wenger (Sandoz Pharmaceutical).

Site-Directed Mutagenesis of CyPA and Cloning of CyPB. Mutations in CyPA were produced by site-directed mutagenesis using a modified rapid polymerase chain reaction (PCR)-based protocol (17). This involves the generation of a PCR fragment using a mutagenic oligonucleotide as one of the PCR primers, and subsequently using this double-stranded fragment as a megaprimer in another PCR reaction to extend the DNA to its entire length. The mutagenised DNAs were then subcloned into pcDNAI/AMP vector and sequenced to verify the presence of the desired mutations and the integrity of the rest of the sequence. cDNAs for the histidine-tagged constructs of α7 and CyP were generated by PCR using an antisense chimeric oligonucleotide containing the coding sequence of six histidines (three glutamines and six histidines) and a stop codon attached to the coding sequence of the five C-terminal amino acids as the reverse primer. The forward primer included the Kozac sequence and the start codon.

To perform coexpression experiments with CyPB, we cloned this CyP from a λgt11 PC12 (rat pheochromocytoma) cDNA library using PCR. The PCR primers were designed from the sequence of rat kidney CyP-like protein that was cloned earlier by Iwai and Inagami (18). We find that the nucleotide sequence of our CyPB clone differs slightly from the Iwai and Inagami kidney clone. However, the deduced amino acid sequence of our clone shows a significant increase in the degree of sequence identity with human CyPB that was cloned by Price *et al.* (19).

Nickel Affinity Matrix Purification. Ten to fifteen oocytes injected with a hexahistidine-tagged construct (α7his or CyPA-his cDNA) or an equal number of those injected with untagged DNA were solubilized in a 200–300 μl of 1% Triton X-100 containing 50 mM of Na_2HPO_4 buffer (pH 7.5, 1 mM phenylmethylsulfonyl fluoride), in a manual homogenizer on ice. The homogenates were centrifuged at 12,000 rpm for 2 min at 4°C. The supernatants were then mixed with equal volume of binding buffer (50 mM Na_2HPO_4/150 mM NaCl, pH 8.0). A third of each sample was saved for later use as starting material for SDS/PAGE. The remaining two-thirds of each sample was mixed with 50 μl of nickel nitrilotriacetate (Ni-NTA) resin (Qiagen, Chatsworth, CA), and left shaking at 4 °C for $>$ 1 hr. It was then centrifuged at 12,000 rpm for 2 min at 4 °C. The supernatant was labeled as flow through and saved. The Ni-NTA matrix was subsequently subjected to several steps of washing and centrifugation at 4°C in the following order: binding buffer, 5 mM of imidazole in high salt buffer (50 mM Na_2HPO_4/300 mM NaCl/1 mM phenylmethylsulfonyl fluoride, pH 8.0), 20 mM of imidazole in high salt buffer, and 100 mM of imidazole in high salt buffer. Elution of bound proteins was done with 500 mM of imidazole in high salt buffer.

Depletion of CyPA by binding to CsA-affinity matrix was performed as follows. Supernatants from detergent-extracted oocytes injected with CyPA or CyPA mutants were prepared as above. They were mixed with 50 μl of CsA–affigel (Pierce) matrix and left shaking for $>$1 hr at 4°C. The supernatant was then used as CyPA-depleted material.

SDS/PAGE Analysis. Equal volumes (80 or 100 μl) of starting materials and fractions from affinity matrix purifications were resolved on 12.5% acrylamide gels. The total amount of protein in all of the purification fractions was estimated to be approximately twice the amount of protein in the starting material. Gels were transferred to nitrocellulose membranes by electroblotting at 4°C. The blots were then treated with either an affinity-purified rabbit CyPA anti-peptide polyclonal antibody ($\approx$5 μg/ml) or sheep anti-α7 polyclonal antibody ($\approx$10 μg/ml). The anti-α7 antibody was raised against bacterially expressed N-terminal domain of α7 by D. Chen in our laboratory. The immunoreactivity was detected by chemiluminence following a peroxidase reaction using an ECL detection kit and exposure to Hyperfilm-ECL (Kodak).

RESULTS

In our previous paper on the effect of CsA on α7 and $5HT_3$ receptor expression in *Xenopus* oocytes, we argued that the CsA-induced reduction in functional receptor expression was unlikely to be mediated through the inhibition of calcineurin by the CsA–CyP complex (2). Calcineurin inhibition is known to underlie the immunosuppressive action of CsA (for review, see ref. 20). It has been shown that CsA analogs that block the PPIase activity of CyPs but do not inhibit calcineurin fail to induce immunosuppression. To conclusively test the involvement of calcineurin in the CsA action on functional receptor expression, we studied the effect of the nonimmunosuppressive CsA analog, SDZ 211–811, on the functional expression of α7 and $5HT_3$ homo-oligomeric receptors in *Xenopus* oocytes. Oocytes were injected with an expression vector (pcDNA or pCDM6XL) containing receptor cDNA, and functional receptor expression was assayed by two-electrode voltage clamp recording of agonist-induced maximal currents on postinjection days 3–5. SDZ 211–811 is a newly synthesized CsA analog that blocks the PPIase activity of CyPA without inducing immunosuppression (21). Oocytes were incubated in Barth's saline containing various concentrations of SDZ 211–811 or the vehicle (0.1% ethanol) throughout the postinjection and prerecording period. Fig. 1*A* shows that α7 receptor-mediated maximal currents were reduced in a dose-dependent manner in oocytes treated with SDZ 211–811. The effective concentration range of this CsA analog is the same as that of CsA (2). This is exactly as predicted because this compound is equipotent to CsA in binding CyP (21). The SDZ 211–811-induced reduction in functional receptor expression was also seen with $5HT_3$ receptors as shown in Fig. 1*B*. These data clearly rule out the possibility that calcineurin inhibition plays any role in CsA-induced blockade of functional homo-oligomeric receptor expression.

We have previously shown that the CsA-induced reduction of functional α7 and $5HT_3$ receptor expression can be reversed by overexpression of rat brain CyPA (2). This result is consistent with the idea that the CsA effect is entirely due to the blockade of this cytoplasmic CyP. However, an alternative explanation might be that the exogenous CyPA merely reduces the effective concentration of free CsA by acting as a high affinity CsA buffer. To test this possibility, we generated a mutant rat brain CyPA that was predicted to have a $\approx$100-fold reduction in its affinity to bind CsA without significant alteration in its PPIase activity. This prediction was based on such an observation in the highly homologous human CyPA by Walsh and coworkers (22). The rat brain CyPA sequence is identical to its human counterpart in the CsA-binding and PPIase active sites. The mutation that renders the CyPA molecule resistant to CsA is a tryptophan to phenylalanine substitution at position 121. The cDNA coding for this CsA-resistant mutant of CyPA was overexpressed in *Xenopus* oocytes at 1:1 ratio with α7 nAChR, and the effect of CsA on functional receptor expression was assayed. As shown in Fig. 2, this mutant produced a complete reversal of the effect of CsA on functional receptor expression. This result clearly rules out the possibility that exogenous CyPA acts as a buffer of CsA in reversing its effect, and lends strong support to the idea that CyPA in the cytoplasm is involved in the maturation of homo-oligomeric receptors. At the very least it demonstrates that the entire effect of CsA on functional receptor expression can be adequately accounted for by the CsA-induced inhibition of CyPA alone.

Because ligand-gated ion channels are integral membrane proteins whose folding and assembly takes place in the ER, it

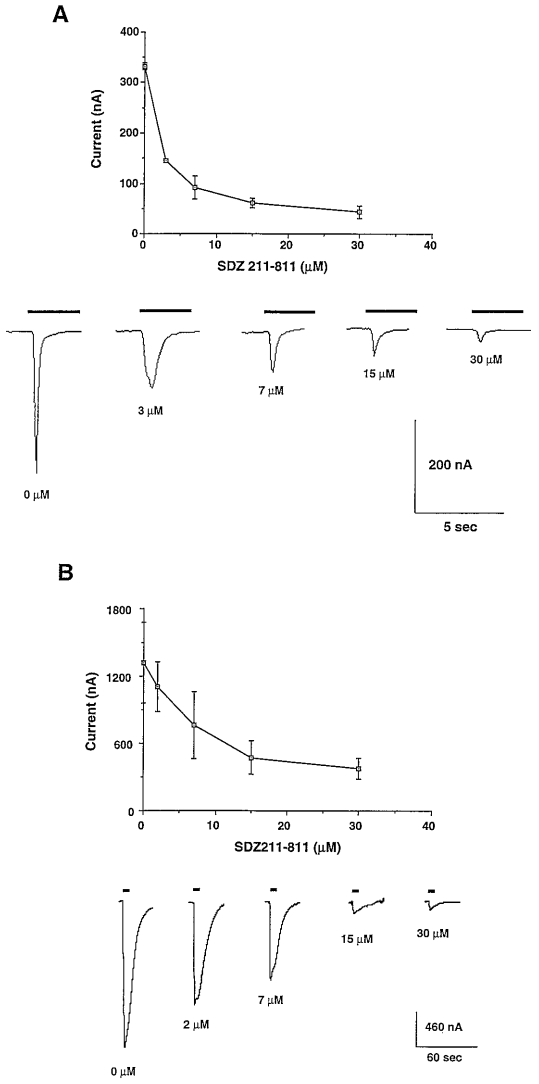

FIG. 1. (*A*) Dose-response curve of the effect of the nonimmunosuppressive CsA analog, SDZ 211–811, on the functional expression in *Xenopus* oocytes of nicotinic α7 homo-oligomeric receptors. Each data point is a mean of near maximal nicotine (250 μM)-induced current responses from 2–7 oocytes from a single representative experiment. The error bars represent standard errors of the means. Example current traces from oocytes incubated in different concentrations of SDZ 211–811 are shown below the graph. The horizontal bars depict the duration of agonist application (≈3 sec). The recordings were carried out at a holding potential of −60 mV. (*B*) Dose-response curve of the effect of SDZ 211–811 on the functional expression in *Xenopus* oocytes of homo-oligomeric $5HT_3R$. Each data point is a mean of near maximal serotonin (10 μM)-induced current responses from 4–6 oocytes from a single representative experiment. Representative traces are shown below the graph. Duration of agonist application was ≈5 sec and the holding potential was −60 mV.

is reasonable to expect that a CyP such as CyPB that is localized in the ER might be playing a role in its maturation. If this is the case then CyPB might be able to substitute for CyPA in reversing the CsA-induced reduction in functional receptor expression. To investigate whether this was true, we first cloned rat CyPB cDNA from a PC12 cell-line cDNA library using PCR with oligonucleotide primers designed from previously cloned rat kidney CyPB sequence (18), and then overexpressed it in *Xenopus* oocytes at a 1:1 ratio with α7 nAChR. Fig. 3 shows the results of such an experiment. CyPB failed to reverse the effect of CsA on functional α7 receptor expression under the conditions in which CyPA showed a reversal. Results similar to the ones depicted in Figs. 2 and 3 were also seen with $5HT_3R$ (data not shown).

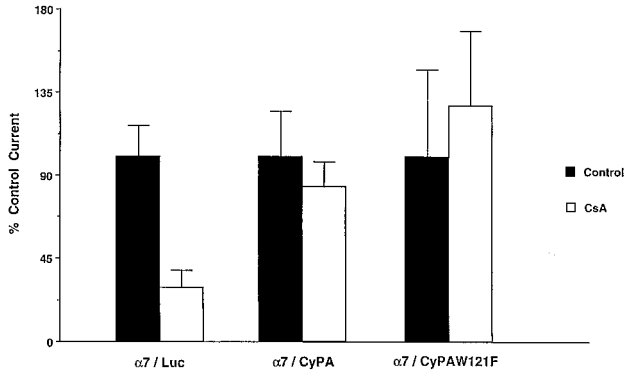

FIG. 2. Effect of overexpression of rat brain cytoplasmic CyP, CyPA, and a CyPA mutant [CyPA(W121F)] lacking high affinity CsA-binding activity on the ability of CsA to reduce functional α7 receptor expression. The open bars represent CsA-treated oocytes (7 μM) [n = 7 for α7/Luc; n = 10 for α7/CyPA; and n = 6 for α7/CyPA(W121F) injected oocytes]. The α7/Luc oocytes are those in which luciferase cDNA in pcDNAI/AMP vector was coinjected with the receptor subunit as DNA dose control. The α7/CyPA and α7/CyPA(W121F) oocytes are those in which CyPA and CyPA(W121F) cDNA in pcDNAI/AMP vector were coinjected, respectively, with the receptor subunit. The filled bars represent vehicle-treated oocytes [n = 7 for α7/Luc; n = 9 for α7/CyPA; and n = 7 for α7/CyPA(W121F) injected oocytes]. The ratio of cDNAs in each coinjection was 1:1.

CyPA is believed to be a multifunctional protein as far as its role in protein folding is concerned. As a PPIase it has been shown to increase *in vitro* the rate of refolding of denatured molecules of proteins such as ribonuclease T1 and human carbonic anhydrase (4, 23–26). On the other hand, it has been proposed that CyPA might function as a molecular chaperone to increase the yield of appropriately folded native protein (ref. 26; see refs. 27 and 28). Because a reduction in functional receptor expression can result from a decrease both in the rate of folding or in the yield of native protein, it is necessary to distinguish between the two possible roles that CyPA might assume in assisting in the maturation of homo-oligomeric receptors. A direct way to test the idea that CyPA might function as a PPIase is to mutate the active site of this enzyme to make it catalytically deficient. We achieved this by substi-

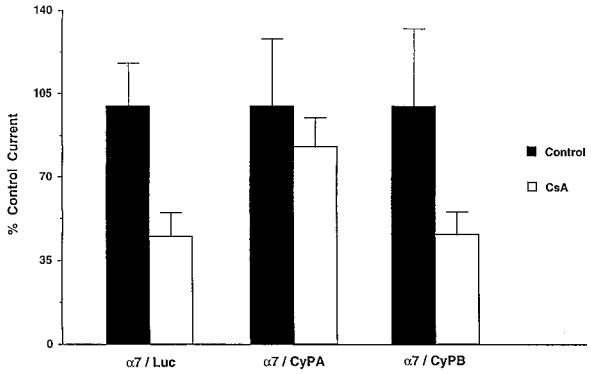

FIG. 3. Effect of overexpression of the ER resident CyP, CyPB on the ability of CsA to reduce functional α7 receptor expression. The open bars represent CsA-treated oocytes (7 μM) (n = 12 for α7/Luc; n = 7 for α7/CyPA; and n = 7 for α7/CyPB injected oocytes). The α7/Luc oocytes are those in which luciferase cDNA in pcDNAI/AMP vector was coinjected with the receptor subunit as DNA dose control. The α7/CyPA and α7/CyPB oocytes are those in which CyPA and CyPB cDNA in pcDNAI/AMP vector were coinjected, respectively, with the receptor subunit. The solid bars represent vehicle-treated oocytes (n = 11 for α7/Luc; n = 6 for α7/CyPA; and n = 9 for α7/CyB injected oocytes). The ratio of cDNAs in each coinjection was 1:1.

tuting an alanine residue for arginine at position 55. This mutation has been shown to cause a 1000-fold reduction in the catalytic efficiency of human CyPA that is identical in amino acid sequence with the rat enzyme in this catalytic region (29). The CsA binding activity of this mutant is not significantly altered. Unlike the wild-type cDNA, cDNA encoding this mutant when overexpressed in *Xenopus* oocytes at a 1:1 ratio with α7 did not produce a reversal of the CsA-induced blockade of functional receptor expression. Fig. 4 graphically depicts this result.

To test whether these data could be due to inadequate expression of the CyPA(R55A) mutant, we directly verified whether this mutant was expressed in *Xenopus* oocytes. This mutant was made such that it had a hexahistidine tag on its C terminus. The wild-type CyPA used in the experiments in Fig. 4 was also similarly tagged. This tag was used to purify these polypeptides by nickel-affinity matrix purification. Fractions from these purifications were then resolved on SDS/PAGE gels and immunoblotted with an affinity-puried CyPA anti-peptide antibody. These immunoblots showed that CyPA(R55A) polypeptide was clearly expressed in oocytes as evidenced from the ≈18-kDa immunoreactive band in the eluate fraction in Fig. 5 *Upper* (lane E). Fig. 5 *Lower* shows that there is no immunoreactive band in the eluate fraction obtained from oocytes expressing untagged wild-type CyPA. This demonstrates that wild-type CyPA does not nonspecifically bind to the nickel matrix, and the band in lane E is mostly likely to be produced by the polyhistidine-tagged CyPA(R55A).

It is possible that although the mutant cDNA is expressed in oocytes, the mutation causes this polypeptide to fold improperly, eliminating not just its PPIase activity but all of its biological activities. If this is true, then mutant CyPA should no longer bind to CsA. To test this prediction we examined whether or not we could deplete the mutant polypeptide from the oocyte lysate in the above experiment by equilibrating it with CsA affinity matrix. As shown in Fig. 5 *Upper* (lane C), mutant CyPA could be completely depleted by such a procedure, suggesting that the expressed CyPA(R55A) protein retains a native-like conformation. These data indicate that the PPIase activity of CyPA is most likely to be essential for functional homo-oligomeric receptor expression.

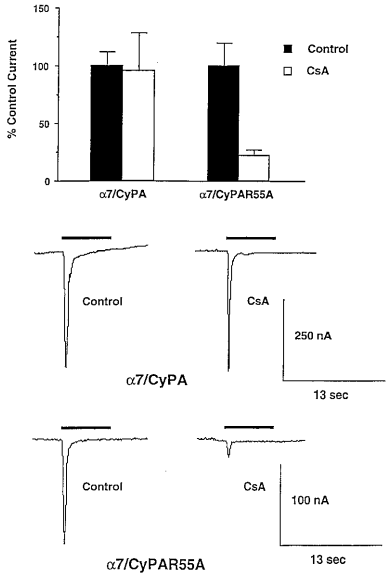

FIG. 4. Effect of overexpression of a CyPA mutant [CyPA(R55A)] deficient in PPIase activity on the ability of CsA to reduce functional α7 receptor expression. The open bars represent CsA-treated oocytes [n = 5 for α7/CyPA injected oocytes and for α7/CyPA(R55A) injected oocytes]. The solid bars represent vehicle-treated oocytes [n = 5 for α7/CyPA injected oocytes and for α7/CyPA(R55A) injected oocytes]. The ratio of cDNAs in each coinjection was 1:1. Both CyPA and CyPA(R55A) used in this experiment possess a C-terminal hexahistidine tag that was used to verify their expression by nickel affinity matrix purification.

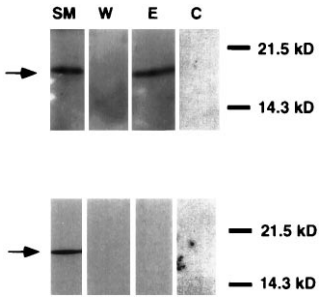

FIG. 5. Western blot of hexahistidine-tagged CyPA(R55A) from *Xenopus* oocytes obtained by Ni-NTA matrix purification. Lanes SM, W, E, and C represent starting material, 20 mM imidazole wash, 500 mM imidazole eluate, and flow through from CsA-affinity matrix purification, respectively. (*Upper*) CyPA(R55A)his-expressing oocytes; (*Lower*) wild-type CyPA-expressing oocytes. Immunoreactivity to a CyPA anti-peptide antibody is indicated by an ≈18-kDa band (arrow) seen only in the starting material and the 500 mM imidazole eluate.

The above results do not rule out the possibility that CyPA might also function as a chaperone of receptor subunits. It is also conceivable that the PPIase and chaperone activities of CyPA share a common active site in which the arginine at position 55 is a key determinant. If either of these cases is true, however, CyPA as a chaperone might form a transient stable complex in association with the nascent receptor subunits. It has been reported that the ninaA protein indeed forms such a stable complex with Rh1 rhodopsin in *Drosophila* photoreceptors (10). When Zuker and coworkers (10) purified a polyhistidine-tagged Rh1 rhodopsin by nickel affinity column chromatography from transgenic *Drosophila* heads expressing this mutant protein, they copurified ninaA protein with it. This result revealed that there was a stable interaction between ninaA and Rh1 rhodopsin.

We performed similar experiments with the α7 nAChR subunit and CyPA. In the first experiment, the α7 protein was tagged at the C terminus with a hexahistidine tag spaced from the α7 sequence by three residues of glutamine. The construction of the cDNA coding sequence for this chimeric protein is described in the *Material and Methods*. This protein was expressed in *Xenopus* oocytes and was found to be functional as determined by two-electrode voltage clamp recordings of agonist-induced currents. Oocytes expressing this construct either singly or in combination with rat brain CyPA (at 1:1 ratio) were solubilized with 1% Triton X-100, and were subjected to nickel affinity matrix purification. The starting oocyte material, flow through, wash (150 and 300 mM NaCl/20 mM imidazole), and elution fractions (500 mM of imidazole) were then subjected to SDS/PAGE and Western blotting with anti-α7 antibody (10 μg/ml). The blots were exposed to Hyperfilm, and then stripped in a 62.5 mM of Tris·HCl buffer (pH 6.7) containing 2% SDS and 100 mM of 2-mercaptoethanol at 50°C for 30 min. After stripping, the blots were incubated in CyPA anti-peptide antibody (5 μg/ml) and then exposed to Hyperfilm. Fig. 6 shows representative examples of these two kinds of Western blots. Fig. 6 shows that α7 protein (≈56 to 58-kDa band, arrow) is eluted in the 500 mM imidazole elution fraction (α7his/CyPA panels). This band is absent in the corresponding fraction in control oocytes expressing $5HT_3$Rs. However, all of the CyPA immunoreactivity is present in the flow through fraction, and none in the α7 elution fraction. The band marked by the arrow was assigned to α7 nAChR subunit by its deduced molecular weight (≈56–58 kDa), and its exclusive presence in the high imidazole

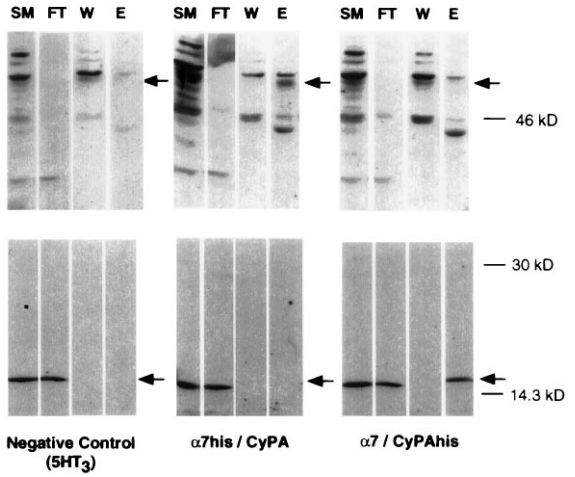

FIG. 6. Western blots of Ni-NTA matrix-purified oocyte material expressing hexahistidine-tagged $\alpha 7$ and wild-type CyPA, hexahistidine-tagged CyPA, and wild-type $\alpha 7$ receptor, or $5HT_3$ receptor. SM, starting material; FT, flow through; W, wash with 20 mM imidazole containing solution; E, elution with 500 mM imidazole containing solution. Equal volumes of material resulting from 10 oocytes in each of the three experiments were loaded in each lane. The top set of blots was probed with a sheep polyclonal anti-$\alpha 7$ antibody. The bottom set was probed with rabbit polyclonal CyPA anti-peptide antibody. The arrows mark the position of $\alpha 7$ immunoreactivity in the upper panels and the position of CyPA immunoreactivity in the lower panels. The negative control blot shown in this figure was made with material from oocytes expressing untagged $5HT_3$ receptors. A detailed explanation regarding crossreactive nonspecific bands seen in the upper panels is given in the text.

elution lane of Ni-NTA matrix-purified material from oocytes expressing polyhistidine-tagged $\alpha 7$ but not any other construct. The polyclonal anti-$\alpha 7$ antibody used in this experiment shows crossreactivity with other nonspecific proteins. The nonspecific bands in the high imidazole lanes are present under all three conditions displayed, including the negative control, although their intensity is weaker in the latter condition. Furthermore, these nonspecific bands were also present in immunoblots performed on uninjected oocytes using a procedure identical to that used in the above experiment (data not shown). The latter was not true of the presumed $\alpha 7$-specific band.

The converse of this experiment was done by coexpressing a hexahistidine-tagged (C-terminal) CyPA with wild-type $\alpha 7$. In this experiment, although CyPA immunoreactivity is eluted in the 500 mM imidazole elution fraction, no $\alpha 7$ immunoreactivity is seen in this fraction (Fig. 6, $\alpha 7$/CyPAhis panels). If a substantial amount of $\alpha 7$ had coeluted with CyPAhis then an $\alpha 7$ immunoreactive band should have been visible in the high imidazole elution lane considering the fact that an $\alpha 7$ immunoreactive band (a band at $\approx$56 kDa at the position, arrow) is in fact present in the wash fraction of the $\alpha 7$/CyPAhis panel. The reduced intensity of the presumed $\alpha 7$-specific bands in the upper $\alpha 7$/CyPAhis panel compared with the upper $\alpha 7$his/CyPA panel from the previous experiment is likely to be due to the highly variable expression of exogenous receptor proteins in oocytes (data not shown). The faint bands seen in the negative control ($5HT_3$R-injected oocytes) at the position corresponding to the $\alpha 7$-specific band may be due to crossreactivity of the antibody with the $5HT_3$R subunit because this band was not seen in immunoblots performed on uninjected oocytes. These results indicate that even under relatively mild purification conditions CyPA does not seem to associate with $\alpha 7$ receptor subunit, a conclusion that is inconsistent with the notion that CyPA forms a stable complex with $\alpha 7$.

DISCUSSION

The above results clearly rule out the possibility that the mechanism underlying CsA-induced reduction in functional homo-oligomeric receptor expression in *Xenopus* oocytes involves the inhibition of calcineurin by the CyP–CsA complex as is the case with immunosuppression. This means that endogenous CyP activity by itself has influence over the maturation of homo-oligomeric receptors. Furthermore, the specific subtype of CyP that is involved appears to be the $\approx$18-kDa cytoplasmic protein, CyPA. The ER-localized CyP, CyPB, is not able to mimic the function of CyPA, although a minor role for this protein in receptor maturation cannot be entirely ruled out. Our data strongly suggest that the endogenous activity of CyPA that is responsible for receptor maturation is likely to be its PPIase activity. However, an additional role for CyPA as a molecular chaperone in this process cannot be entirely ruled out. However, it is unlikely that this role involves a stable complex formation with the receptor subunit.

The observation that the cytoplasmic CyP, CyPA, reverses the CsA effect on receptor maturation while the ER localized CyP, CyPB, does not strongly suggests that the site of CyP action on receptor subunits is at least transiently exposed to the cytoplasmic environment. This makes it unlikely that the large extracellular N-terminal region of the subunits would be a site of CyP action because topologically it would be located within the lumen of the ER. This idea is consistent with an observation that has been recently made in our laboratory (30). This observation is that a chimeric subunit in which the N terminus (up to the beginning of first transmembrane domain) of the muscle nAChR δ subunit is replaced by the corresponding part of the $\alpha 7$ subunit completely rescues the wild-type $\alpha 7$ subunit from the effect of CsA, whereas the reverse chimera does not. The above discussion implies that the most likely candidate sites for CyP action must reside in the cytoplasmic loops between the first and the second, and between the third and the fourth transmembrane domains. It is also possible that the loop between the second and third transmembrane domains, or parts of these transmembrane domains, might be transiently oriented toward the cytoplasm during subunit folding.

A large body of evidence suggests that CyPA functions as a PPIase in the folding of proteins *in vitro* and *in vivo* (31). This enzymatic activity of CyPA has a K_{cat} of $\approx$13,000 per sec, which is more than an order of magnitude greater than that of the other abundant PPIase, FKBP-12 (K_{cat} of $\approx$300 per sec) (32). As far as the chaperone function of CyPA is concerned, there is only one piece of direct evidence in favor of such a role, namely that CyPA increases and CsA decreases the yield of properly folded human carbonic anhydrase in an *in vitro* denaturation/refolding experiment (26). However, these data have recently been reinterpreted in favor of a more traditional PPIase function for CyPA (27, 28). It has been shown that CyPA and ninaA directly stably associate with substrate proteins, HIV gag1 and Rh1 rhodopsin, respectively (10, 33, 34). This association has been construed as evidence that the interaction is chaperone-like. However, in the case of the HIV coat protein, it is clear that this interaction does not lead to an increase in the yield of fully assembled viral particles suggesting that it is unlikely that CyPA is chaperoning the folding of that protein (33, 34).

The lack of a stable association of CyP with a substrate protein as determined by nickel affinity matrix purification of a polyhistidine-tagged mitochondrial CyP has been used recently by Matouschek *et al.* (35) as supporting evidence to propose that a mitochondrial CyP in yeast works as a PPIase rather than a chaperone. In similar experiments with hexahistidine-tagged $\alpha 7$ subunit or CyPA, we were unable to copurify the untagged counterpart. This result, as in the above case, suggests that in receptor maturation CyPA is unlikely to assume the role of a conventional chaperone that requires a

period of stable interaction with its substrate. An alternative possibility, however, is that CyPA does not directly interact with the receptor subunit and instead acts on an intermediate protein that is involved in the folding of the nascent subunit polypeptide. However, a stronger piece of evidence in favor of a role for the PPIase activity of CyPA in receptor maturation is the result of the PPIase active site mutation experiment. The removal of the ability of CyPA to reverse the CsA blockade of functional receptor expression by the (R55A) active site mutation is consistent with a PPIase function for CyPA in this process. This result cannot be explained by a mutation-induced reduction in protein synthesis or misfolding of the expressed CyPA mutant protein. First, this is because immunoblotting experiments showed no reduction in immunoreactivity corresponding to the expressed mutant protein. Second, this immunoreactivity could be completely depleted from fractions that were adsorbed to a CsA affinity matrix before immunoblotting. The latter experiment suggests that the mutant CyPA protein was folded in a native-like conformation because it exhibited high affinity binding to CsA.

In conclusion, our experiments present some clear pieces of evidence in favor of the involvement of the PPIase activity of CyPA in the cytoplasm in the maturation of $\alpha 7$ homo-oligomeric receptors. We also provide evidence that this process may not require a detectable stable interaction between CyPA and receptor subunits. Since these results could be extended to the $5HT_3$ homo-oligomeric receptor, it is likely that this mechanism is a general maturational mechanism that operates during the functional expression of homo-oligomeric ligand-gated ion channels.

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